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<p>Previous studies on fishes have investigated the effects of sounds on only one or two of the otolithic endorgans of fishes. In addition, these studies included only species that are considered to have specializations that enhance their hearing capabilities. In the present study we extend our earlier work to include pulsed as well as continuous tones and an examination of all the endorgans in the ear (saccule, utricle, lagena, cristae of the semicircular canals) and of the hair cells of the other teleost mechanoreceptor, the lateral line. We demonstrated that intense sounds will have minimal damage on the structure of the fish ear, but we suggest that higher intensity sounds could do severe damage if presented for several hours.</p>			
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Final Technical Report

ONR Grant N00014-92-J-1889 Effects of sound on hair cell receptor systems in fishes

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ABSTRACT

Fish (*Astronotus ocellatus*, the oscar) were subject to pure tones in order to determine the effects of sound at levels typical of man-made sources on the sensory epithelia of the ear and the lateral line. Sounds varied in frequency (60 or 300 Hz), duty cycle (20% or continuous), and intensity (100, 140 or 180 dB re: 1 μ Pa). Fish were allowed to survive for 1 or 4 days post treatment. Tissue was then evaluated using scanning electron microscopy to assess the presence or absence of ciliary bundles on the sensory hair cells on each of the otic endorgans and the lateral line. The only damage that was observed was in four of five fish stimulated with 300 Hz continuous tones at 180 dB re: 1 μ Pa and allowed to survive for four days. Damage was limited to small regions of the striola of the utricle and lagena. There was no damage in any other endorgan, and the size and location of the damage varied between specimens. No damage was observed in fish that had been allowed to survive for one day post stimulation, suggesting that damage may develop slowly after exposure.

INTRODUCTION

Intense sounds can destroy sensory hair cells of the mammalian and avian inner ear (e.g., Spoendlin, 1971; Engström et al., 1986; Saunders et al., 1986; Corwin and Cotanche, 1988) and lead to permanent loss of hearing (e.g., Liberman et al., 1986). However, while such damage is well documented for certain mammalian model species and for humans (see papers in Salvi et al., 1986), little is known about the effects of intense sound on many other species, and particularly those that have not been domesticated (e.g., Fletcher and Busnel, 1978; Kryter, 1985).

Among wild animals, investigations on the effects of intense sound on marine organisms have been particularly limited (NRC, 1994). Of the few studies on marine organisms, most have been directed at determining behavioral responses to sound rather than determining damage to the sensory receptors. Possibly the best data of this type are on marine mammals, which show that gray whales will avoid continuous underwater sounds of about 120 dB (re: 1 μ Pa) (Malme et al., 1983, 1984). This behavioral response is, however, most likely an avoidance of a bothersome stimulus rather than avoidance of a painful signal.

Studies on fishes are also very limited. In one behavioral study on the goldfish (*Carassius auratus*), Popper and Clark (1976) demonstrated that four-hour exposures to 149 dB (re: 1 μ Pa) sounds at 300, 500, 800 and 1,000 Hz caused temporary threshold shifts lasting two to four hours, but there was complete recovery from this stimulation even after repeated exposure to the sound during daily experiments for several days or weeks. In that study there was no behavioral evidence for permanent hearing loss that would potentially have been a sign of hair cell damage in the ear or lateral line.

Two additional studies, however, lead to the suggestion that some intense sounds result in limited damage to sensory hair cells in the ears of fishes. Cox (aka Hastings) et al. (1986a, b; 1987) stimulated goldfish with pure tones at 250 Hz and 500 Hz for two hours at sound pressure levels of 204 dB and 197 dB (re: 1 μ Pa), respectively. Examination of the otolithic endorgans revealed some loss of ciliary bundles from sensory hair cells in two of the endorgans, the saccule and lagena, indicating hair cell damage (e.g., Spoendlin, 1971; Engström et al., 1986; Saunders et al., 1986). Enger (1981), studying the cod (*Gadus morhua*), found that exposure to 180 dB (re: 1 μ Pa) sounds for one to five hours at several frequencies from 50 Hz to 400 Hz destroyed ciliary bundles on sensory cells of the saccule in a pattern that had some suggestion of a crude frequency dependent pattern of damage along the sensory epithelium. Finally, a study by Denton and Gray (1993) demonstrated that intense displacement stimulation will result in damage to the hair cells of the lateral line of clupeid fishes (herrings and relatives) resulting from overstimulation of the cupula that overlies the hair cells, resulting in overstimulation of the hair cells themselves.

Previous studies on fishes have investigated the effects of sounds on only one or two of the otolithic endorgans. In addition, these studies included only species that are considered to have specializations that enhance their hearing capabilities. In the present study we extend our earlier work (Cox et al., 1986a, b; 1987) to include pulsed as well as continuous tones and an examination of all the endorgans in the ear (saccule, utricle, lagena, cristae of the semicircular canals) and of the hair cells of the other teleost mechanoreceptor, the lateral line. In addition, the acoustic stimulus in this study was a plane traveling wave instead of a standing wave as used in our previous work. The species selected for study was the oscar, *Astronotus ocellatus*, a member of the family Cichlidae.

This species was chosen since it, like the majority of fishes, does not have morphological specializations to enhance hearing capabilities (Popper and Fay, 1993). Behavioral studies have shown that the oscar can detect sounds from below 200 Hz to about 900 Hz, with best sensitivity at 200 Hz of 118 dB re: 1 μ Pa (Yan and Popper, 1992).

I. METHODS

This study was performed at the Ohio State University (OSU) and the University of Maryland at College Park (UMCP). Fish were stimulated with sounds at pressure levels typical of man-made sources in a waveguide at OSU. The fixed tissue was then shipped overnight to UMCP where it was processed for scanning electron microscopy (SEM) and evaluated.

The experiment were done 'double-blind.' The fish were coded to hide treatment groups at OSU before sending to UMCP. The investigators at UMCP did not give feedback on the results of the SEM evaluation until the full series of experiments were complete and all of the data analyzed. After completion of the first set of experiments, a second smaller set was done at select frequencies and intensities to confirm initial findings. These studies were also done using the double-blind paradigm.

A. Experimental Setup

The experimental apparatus used at OSU consisted of a Plexiglas[®] waveguide approximately 15 meters long with an inside diameter of 0.12 meters. The waveguide was flanged to an NRL USRD J9 underwater sound projector with a bandwidth of 40 to 20,000 Hz. The apparatus was placed on wooden supports, submerged in a water-filled channel approximately 1.2 meters deep, and filled with water. A sine wave generated by the source function on an HP 35665A digital signal analyzer was fed through an audio amplifier into the J9. The removable test section of the waveguide in which the fish was positioned was located approximately 3.8 meters from the face of the J9. A hydrophone (B&K 8103) was used to monitor the acoustic pressure just downstream from the the fish during exposure to sound. The hydrophone signal was recorded by the HP 35665A. A personal computer with an HP-IB (IEEE-488) interface to the digital signal analyzer was used to control the source and automatically store the pressure data.

B. Waveguide design

Because of the large chamber size required for anechoic or reverberant fields in water (ANSI S1, S2; Broch, 1971; Koidan and Hruska, 1978), a long, flexible waveguide was used to create a traveling wave for this study. The diameter of the cylindrical waveguide allowed only plane wave propagation along its longitudinal axis. The flexible waveguide material (Plexiglas[®]) reduced the effective stiffness (relative to water), and thus the speed of sound and wavelength of the acoustic disturbance. In addition, the energy of the wave was dissipated as it traveled along the waveguide by the local motion of the cylindrical wall. Thus little, if any, reflection occurred at its end. (Reflections are undesirable because they would create a standing wave rather than a traveling wave in the waveguide.) At the location of the animal, (3.8 m from the source), a traveling wave occurred at 300 Hz, but some back reflection at the end of the waveguide was present at 60 Hz. The pressure distribution at 60 Hz indicates the formation of a partial standing wave as the end of the waveguide is approached; consequently, the local acoustic particle velocity at the location of the animal was slightly different than that of the plane wave value (p/pc).

C. Experimental Animals

Oscars were obtained from a local supplier in Columbus, Ohio. Fish were 5 to 9 cm standard length in order to insure that they had approximately the same number of sensory hair cells (Popper and Hoxter,

1984). The fish were maintained in a community aquarium in temperature-controlled, filtered water until they were used in the experiments. All experiments were approved by the Animal Care and Use Committees of both OSU and UMCP.

D. Test Matrix

The total experiment consisted of 64 animal trials, including 5 controls. The controls were treated exactly the same as the test animals except that no sound was played when they were placed in the waveguide. Table I provides a summary of the experimental parameters. At least 2 animals were exposed at each parameter in combination with each of the other parameters for 1 hour.

E. Procedure

Each fish was placed in approximately 1 gallon of water from the community tank in which it had been kept until used in the experiments. Over a half-hour period, water from the waveguide was gradually added to displace that from the community tank. This was done to equilibrate the fish to the temperature and the water chemistry of the waveguide. Next, the fish was placed in a small cylindrical cage made of PVC mesh which has an acoustic impedance nearly the same as water. The cage was suspended in a removable test section of the waveguide which was then flanged back into the waveguide so that the fish was facing the sound source so that the fish was facing the sound source. Each animal was located approximately 3.8 meters downstream from the J9. The fish were not anesthetized during the procedure and did not show signs of stress during the one-hour test.

After exposure to the sound, the fish were transferred (again over a half-hour to avoid an abrupt change of temperature and water chemistry) to a ten gallon aquarium and held for 1 or 4 days to assess damage over time. They were then sacrificed with an overdose of MS-222 (an anesthetic for poikilothermic vertebrates). The heads were quickly opened and fixative (3-4% glutaraldehyde in phosphate buffer) was injected into the cranial cavity. In addition, two sections of the lateral line, one from the trunk and one from the head, were removed and placed in fixative. After several hours the tissue was placed in fresh fixative and shipped overnight to UMCP.

At UMCP, the tissue was further dissected to expose the sensory surfaces of the ears and the lateral line. The tissue was post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer and dehydrated through a series of ethanols prior to critical point drying using CO₂ as the intermediary fluid. The tissue was mounted on stubs, vacuum coated with gold-palladium, and viewed on an AMRAY model 1820D SEM. Each piece of sensory epithelium was documented with video prints. Prints were evaluated independently by two investigators. In all cases, there was complete agreement as to the state of the tissue between the investigators.

II. RESULTS

Control animals had no loss of ciliary bundles in any of the endorgans studied. Some variation, however, was found in bundle structure and condition. Experimental tissues were, with a few exceptions described below, very similar to controls. Ciliary bundle damage in experimental tissue was usually within the normal variation found in controls. In a few cases (no more than 10 epithelia from all of the fish), the damage covered an area that took the shape of the tip of a forceps used to remove the otolith (long and narrow regions of damage), or was in close proximity to major breaks in the tissue. In all cases, these types of damage were readily and independently identified by both individuals who evaluated the tissue.

In contrast, four of five animals that were stimulated at 300 Hz with a 180 dB (re: 1 μPa) continuous wave signal for one hour and allowed to survive for four days had a small amount of damage in the utricle and/or lagena of at least one ear (Table II). This damage could not be explained in terms of mechanical manipulation of the tissue or normal variation in controls. In each case, damage was limited to small portions of the striola regions of the lagena and utricle. The striola is a narrow band of cells near one edge of the utricle and down the center of the lagena that has relatively small ciliary bundles and hair cells oriented in opposite directions. In only one fish (OS080933) did damage occur in both ears, and in no case was damage greater than approximately 15% of the total area of the epithelium. Undamaged tissue from these animals closely resembled the tissue from other experimental and control animals.

Damage was never encountered in the lateral line or the saccule or cristae of the ear. Moreover, damage was not found in fish allowed to survive for four days after exposure to 180 dB continuous wave

signals at 60 Hz or to fish exposed at 180 dB to 20% duty cycle signals at 300 Hz. In addition, we found no damage in fish that had been allowed to survive for only one day after exposure to any of the 180 dB sounds.

III. DISCUSSION

Our results show that sounds as high as 60 dB above threshold do no fully replicable damage to the sensory hair cells of the ear and lateral line of the oscar. The only damage seen was found in four of five fish that received maximum stimulation (one-hour 300-Hz continuous tone, 180 dB re: 1 μ Pa) and allowed to survive 4 days post stimulation. Three of these fish had a small amount of ciliary bundle loss in one ear, while the fourth fish had a small amount of damage in both ears (Table II). Damage in all four fish was restricted to a small region of the utricle or lagena, but the region damaged, and the extent of damage, differed in each ear. Other cells near the regions of damage looked totally normal and like controls or other experimental animals. There was no indication of damage to the ciliary bundles of the semicircular canal cristae or to those of the regions of the lateral line of any animals studied in this investigation. Moreover, signals of similar acoustic pressure and frequency, but with a 20% duty cycle, and all 60 Hz signals, did not have the same effect on hair cells.

The damage encountered in these four fish is likely to be the result of acoustic stimulation, although an alternate possibility is that this damage resulted from dissection or other aspects of tissue preparation. While this damage is not consistent with that seen from using forceps, it is possible that the damage occurred when the otolith membrane, which lies in tight opposition to the epithelial surface, was removed. The striola region, the area where we encountered virtually all of the damage, is usually the part of the utricular and lagena epithelia most tightly adhered to by the otolith membrane, and so removal of the membrane could have potentially caused damage. However, we would argue against this possibility since similar damage was not seen in any fish receiving different stimulus parameters. If damage occurred during removal of the otolith membrane, it is reasonable that such damage would have been encountered in at least some of the other fish.

Our general conclusion is that the highest level tones used in this study caused some, but limited, hair cell damage in the ears of the oscar. The damage that did occur was consistently in the striola region of the epithelia. The significance of the damage in the striola is not clear, although this may be the region of the utricle and lagena that is most sensitive to motions of the otolith during sound stimulation. Interestingly, it is the striola region of these two endorgans that is most sensitive to damage by ototoxic drugs (Yan et al., 1991; Lombarte et al., 1993) and these cells may be more sensitive to trauma than other cells on the epithelium. In fact, striolar hair cells are quite different morphologically from other hair cells on the epithelium of these endorgans (Chang et al., 1992), and damage may be correlated with the particular cell structures found in these cells.

The lack of damage in the cristae and lateral line could be related to their having overlying cupular structures rather than otoliths, and the fact that both of these groups of endorgans are more responsive to very low frequency signals than the otolithic endorgans (e.g., Popper and Platt, 1993). Why we encountered no damage in the saccule is not known. Interestingly, the saccule of the oscar has some hair cells that are morphologically similar to those of the striola of the utricle and lagena (Popper et al., 1993), and these were not damaged.

A. Repair and Time Course of Damage

One possible explanation for the limited hair cell loss in our experimental animals is that the cells were regenerated very quickly after damage. Hair cell regeneration is known to occur in the ear and lateral line of fishes treated with ototoxic drugs (Yan et al., 1991, Lombarte et al., 1993; Song et al., in press) and regeneration occurs in birds following intense sound stimulation or drug treatment (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Thus, it is reasonable to expect that fish would regenerate hair cells after sound damage. Still, in studies using ototoxic drugs in the oscar, observable damage was present before four days post-treatment, while recovery of the sensory epithelium did not occur for seven or more days post-treatment. Consequently, we feel that it is unlikely that the survival times used in the present study were long enough for regeneration to occur.

A second possible explanation for the low levels of hair cell damage observed here is that the time for such damage to occur after tonal stimulation may be considerably greater than that for damage by ototoxic drugs. Longer survival times may thus be necessary for acoustic damage to be manifested as hair cell or ciliary

bundle loss. Thus, even at four days post-treatment we may have only been seeing the initial stages of hair cell damage, and this damage may have continued to develop had we allowed for longer survival times.

Clearly, future evaluations of damage due to sound stimulation should allow fish to survive for several days post stimulation in order to see the effects of stimulation. The long term effects of sound damage have particular consequence in cases where relatively high sound levels are used to modify the behavior of fish (e.g., Dunning et al., 1992; Ross et al., 1993; Kundsen et al., 1994). While immediate effects (24 hours or less) may not be apparent, damage to the ear or lateral line may increase over time. Conversely, it is possible that such damage may be alleviated by the regeneration of the sensory epithelium over time if the initial damage is not too severe. Of course, alterations in behavior produced as a consequence of not having a portion of their hair cells for a period of time following sound stimulation could be as devastating to the survival of a fish as would be permanent damage to the hair cells!

B. Comparisons to Earlier Fish Data

Earlier studies on cod and goldfish did demonstrate some damage to sensory epithelia caused by high-level sounds. Enger (1981) used 180 dB (re: 1 μ Pa) signals from 50 to 400 Hz to damage the ears of cod (*Gadus morhua*). He found that these signals, which are about 100-110 dB above threshold in the 150-250 Hz band (the most sensitive for this species), would cause repeatable damage (loss of ciliary bundles) in animals that were exposed to sounds 1 to 5 hours before sacrifice. Enger's study included 26 test animals and four controls.

Cox et al. (1986a, b; 1987) exposed 28 goldfish to 250 Hz continuous pure tones at 189, 192 and 204 dB (re: 1 μ Pa) and to 500 Hz pure tones at 182, 192 and 197 dB (re: 1 μ Pa) for approximately two hours. These sound pressure levels are about 120-140 dB above threshold for the goldfish at these frequencies. The study also included eight controls. The fish were sacrificed immediately following their exposure and then handled in much the same way as the current study; however, only sensory epithelia from the saccule and lagena were examined. Destruction of ciliary bundles was found to correlate with increasing sound pressure level at a 95% confidence level. Damage occurred in none of the fish at 182 dB re: 1 μ Pa (500 Hz) but increased to 100% of the fish at 204 dB (250 Hz).

The differences in results obtained for the oscar as compared to those for the goldfish and cod could be explained by a number of factors including differences between species or differences in experimental parameters. The most compelling possibility is the relationship between the sound levels used in the experiments and the thresholds of hearing in different species. In the case of both cod and goldfish, the sound levels used in the experiments were 90 to 140 dB above the threshold for these species. While the actual sound pressure level used for the oscar was about the same as that used for the cod, the thresholds of the oscar at frequencies near that of the test frequencies are substantially higher than for the cod (Yan and Popper, 1992). In fact, the test signal was only 60 dB above threshold at 300 Hz. Remarkably, some damage occurred even at only 60 dB above threshold. This could be due to the fact that in this study the sound field was a plane traveling wave instead of a standing wave as was the case in previous experiments with fish and cod. Both Cox et al. (1986a, b; 1987) and Enger (1981) placed fish at a point of maximum pressure and minimal particle velocity in their test apparatus, thus minimizing the effect of acoustic particle motion. The current results indicate that acoustic particle acceleration is an important stimulus parameter since for a harmonic plane wave, it would increase proportionally with frequency. Thus for the oscar, the acoustic particle acceleration at 300 Hz was about five times that at 60 Hz for the same SPL. (For a harmonic plane wave, the acoustic energy density is proportional to the square of the pressure amplitude and would not change with frequency.) This could explain why some damage was observed at 300 Hz and not 60 Hz, and at only 60 dB above the oscar's pressure threshold for hearing. It must also be recognized that the results for all three species are limited to just a few frequencies, and all of the signals tested for each of species were pure tones. It is not known whether MORE damage would have occurred in the oscar at other frequencies closer to 200 Hz, the best sensitivity of this species (Yan and Popper, 1992).

C. Extrapolation to Other Species of Teleost Fishes

It is unclear whether these results are relevant to other species. At this time, any extrapolation must be done with caution since the data for each of the three species studied to date are very limited. Moreover, the species are taxonomically very distinct from one another and there are significant differences in the structure

of their peripheral auditory systems.

Still, the effects of sound on the ears of teleosts is a question of considerable practical consideration. Sound has been proposed as a method to control the movements of fish, with particular interest in using sound to keep fish from water diversions or from the intakes of hydroelectric dams and power plants (e.g., Dunning et al., 1992; Ross et al., 1993; Kundsen et al., 1994). It is not clear, however, whether the sounds proposed for such uses would have adverse effects on fish. While the results from the oscar study may potentially be extrapolated to effects on other fishes that are not specialized for hearing (e.g., salmon, tuna, flatfish), the effects on those species must be made with caution. In many cases, the ears of fishes that have specializations for hearing (hearing specialists, such as the goldfish and cod) are quite different from fishes that do not hear well (hearing generalists), and the pathway for sound to the ear and the way that stimulation may occur could differ considerably (e.g., Popper and Fay, 1993).

If it is necessary, as discussed above, to generate sounds 90 to 140 dB above threshold to always damage fish inner ear sensory epithelia, we would suggest that it would require sound levels of at least 220-240 dB re: 1 μ Pa at 300 Hz to potentially produce more extensive damage to sensory hair cells in fishes that are not specialized for hearing. These high sound levels would be needed for hearing generalists since their thresholds much closer approximate those of the oscar than thresholds of the goldfish or cod. Such high intensity signals would require substantial energy to generate and so are probably well beyond the levels that might be generated in the field (e.g., around a hydroelectric dam) to control fish movement. Thus, it is likely that any sounds used to control the behavior of hearing generalists are unlikely to damage the ear or lateral line in these species. At the same time, signals of 180 dB or even less, as used in some field studies (Dunning et al., 1992; Ross et al., 1993; Kundsen et al., 1994), could potentially damage the ear in hearing specialists if the fish remained in the area of sounds of this level for an extended period of time.

IV. CONCLUSIONS

This study is the first to examine the effects of pure tones on each of the sensory surfaces of the ear and lateral line in a teleost fish that is not specialized for hearing. The results indicate that continuous wave or pulsed sounds of one-hour duration at sound pressure levels up to 60 dB above threshold cause little repeatable damage to sensory hair cells of the otolithic endorgans in the oscar. The only exception was that one hour of continuous stimulation at 300 Hz and 180 dB produced some limited damage, but this was limited and inconsistent.

Considerable caution should be taken in extrapolation of these results to other signals and other species, and future evaluations of the effects of sounds should include longer recovery times. Perhaps more importantly, the results suggest that short term stimulation with sound (e.g., for minutes) or stimulation when fish are free to leave the site of stimulation (unlike the condition in these experiments) may have even less of an effect on the ear or lateral line. Still, it is possible that these shorter duration sounds are having adverse effects on other systems of the fish's body, and so our results cannot be interpreted to indicate that sound is not damaging as long as it is of shorter duration, or lower intensity, than used in this study.

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Table I: Experimental parameters.

Days Post Treatment	Frequency (Hz)	Pulse Duration	SPL (dB re: 1 μPa)	Number of Animals
Experiment 1				
1 4	60 300	continuous wave 20% of 1 minute	100 140 180	54
			ambient	
1 4	none	NA	ambient	5
Experiment 2				
1 4	300	Continuous wave 20% of 1 minute	180	5

Table II: Summary of effects of stimulation on the five fish that received 300 Hz continuous stimulation for 1-hour at 180 dB and then were allowed to survive for four days before sacrifice.

Fish Number	Endorgan(s) damaged	Extent of damage
OS042693	Right lagena	Small patch in striola (<2% area of epithelium and <5% of striola)
OS080993	Left utricle	Elongate region near striola (<15% of epithelium but about 30% of striola)
	Right utricle	Small patch in striola region (<5% of epithelium)
	Right lagena	Patch in striola region (<10% of epithelium)
OS071994	No damage	
OS070594	Left utricle	Very tiny patch in striola (<1% of epithelium)
OS071194	Right lagena	Small patch (<10% of epithelium)
	Right utricle	Two small patches of striola (together less than 2% of epithelium and <5% of striola)
	Right lagena	Small patch in striola (<5% epithelium)

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- Hastings, M. C., Finneran, J. J., Popper, A. N. and Lanford, P. L. (1994). Determining the effects of low-frequency sound on the fish auditory system. *J. Acoust. Soc. Am.* 96: 3297.
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